IMPROVED METHOD FOR AVOIDING THE DEGRADATION OF AN ACTIVE PRINCIPLE

The present invention relates to an improved method of avoiding degradation of an active principle.

Whether it be in cosmetics, in pharmacy, in detergents, or indeed in the food industry, a product generally owes its functionality to the presence of a molecule of "active material" or "active agent". By way of example, mention can be made of vitamins which are used in the food industry and in pharmacy, of enzymes which are used in detergents, of organophosphates used as insecticides, or indeed the aromas and fragrances used in sanitation, the food industry, or cosmetics.

One of the essential characteristics of an industrial or pharmaceutical product, apart from its activity and its effectiveness, is its stability, whereby a product is obtained that has a lifetime which is satisfactory. Unfortunately, numerous active materials are molecules that are particularly fragile, and that degrade under the effect of environmental constraints too quickly for the lifetime that is desired of the product containing them. This applies to certain vitamins such as vitamins C, A, or E, to many enzymes such as proteases, for example, and more generally to many proteins and biological molecules or indeed in the field of insecticides, of malathion, and of pyrethrinoides, and more generally, of reducing or oxidizing molecules.

Numerous strategies have been devised to avoid the degradation of such fragile active molecules. These strategies depend on the nature of the reactions that give rise to degradation. The reactions most frequently involved are either hydrolysis, or else oxidation and reduction. Other, more specific, reactions also take place, such as autoproteolysis for proteases.

When water is a direct or indirect cause of degradation, a simple solution consists in avoiding contact between the active molecule and an aqueous

medium. This applies for example to insecticides dissolved in an organic solvent and packaged in an aerosol. Unfortunately, this option is not always available, for example with cosmetics or in the food industry where, for obvious reasons, it is not possible to use organic solvents. Furthermore, for reasons of ecology and of public health, the present trend is to eliminate the use of organic solvents in all branches of industry.

When water is merely an indirect cause, e.g. by oxidation due to dissolved oxygen, it is possible to work with degassed water. More generally, it is possible to work in an inert atmosphere, both during manufacture and for conservation of the final product. This solution is often used in the food industry where vitamin-enriched liquids are packaged either in an air-free vacuum, or under an inert atmosphere. Unfortunately, that method serves to limit degradation only until the container has been opened. It is therefore not suitable for products that require a long lifetime after opening.

Enzymes are proteins which catalyze chemical reactions in highly specific manner. They are in very widespread use in industry, e.g. in detergents for degrading proteins (proteases), lipids (lipases), or amyl-containing residues (amylases), thereby making cleaning easier. Such proteins are generally unstable in solution, and they are therefore used above all in powder form. Their use in liquid form (whether for washing fabrics or for washing dishes) is highly limited because of their long-term instability.

Similarly, a present trend is towards using enzymes in cosmetics, e.g. proteases for aid in peeling the skin, and thus for renewing it. In this case also, the instability of enzymes prevents them from being simple to use.

Immobilizing enzymes is a very well-developed subject, at least in research, given that industrial

applications are not yet very numerous. Work on encapsulating enzymes forms a part of the work being done in the field of bioencapsulation which also includes encapsulating "living" organisms (yeast, bacteria, ...). In general, use is made of polymers that form a matrix that immobilizes the enzyme.

In those pieces of work, the objective is generally to immobilize the enzyme (or the yeast) so as to have it in a form that is easy to manipulate, e.g. in the form of microbeads that can be extracted from the reaction medium after use merely by filtering or screening depending on size. Consequently, the encapsulated form of the enzyme must retain its activity. The encapsulation material must be sufficiently porous to allow the substrates and the reaction products to diffuse therethrough. A review of such developments can be found in "Bioencapsulation in biotechnology", Biomat., Art. Cells & Immob. Biotech., 21, 291-297 (1993).

The main reactions leading to a loss of enzyme activity are chemical degradation, loss of three-dimensional shape, or a plurality of enzymes aggregating. The methods typically used for avoiding or limiting excess degradation are immobilization and chemical modification of the molecule.

Protecting enzymes from being denatured by encapsulating them is less common. Since the polymer matrices used for immobilization allow substrates to diffuse through, they are too porous for providing the enzyme with real protection. The main degradation reactions are those that affect proteins. These include in particular specific hydrolyses of bonds between amino acids and reactions leading to denaturation by a change of conformation that do not involve covalent bonds but that cause the enzyme to lose activity by modifying accessibility of its catalyst site, for example. In the article "Prolonged retention of cross-linked trypsin in calcium alginate microspheres", J. Microencapsulation,

14, 51-61 (1997), it is shown that it is necessary to begin by cross-linking the enzyme by means of glutaraldehyde in order to ensure that it is protected, with that causing 50% of its activity to be lost.

With enzymes, e.g. in washing liquids or in cosmetic creams, it is not possible to use a non-aqueous solvent, both for reasons of safety and for reasons of cost. Introducing enzymes into products of these types therefore encounters real difficulties. One solution that is sometimes used in cosmetics, consists in using sophisticated packaging made up of two independent tanks, one containing the active molecule and the other containing the remainder of the preparation. Mixing is performed extemporaneously at the moment when the product is used by means of a system of dual metering pumps on the flask. That solution is quite expensive and awkward to use. That solution has also been used in cosmetics to make beauty products available that contain vitamins.

Another method consists in separating the active agent from its medium, but to do so on a microscopic scale by microencapsulating it in polymer microspheres, or by coating it, e.g. using a fluidized bed technique to coat it in a polymer matrix. That technique can be advantageous, in particular for products that are to be incorporated in dry preparations. It suffers from the drawback of requiring the polymer coating or shell to be broken to release the active agent. It is therefore poorly adapted to cosmetics where the active agent must be released spontaneously on application of the product, or to food products which must release the active agent in the mouth.

When the active material is moderately unstable, it can be advantageous to use protective molecules. This applies to anti-oxidizing agents that are in widespread use in cosmetic creams and in foodstuffs. In general they are merely added to the preparation, but because of dilution they need to be added thereto in quantities

larger than necessary for obtaining an effect. Furthermore, as with many additives, use thereof is becoming subject to more and more regulation, and the authorized levels are being decreased.

It is known to encapsulate active principles in vesicles based on surfactants.

In general, such vesicles have one or more bi-layers. We use the term "unilamellar" to designate vesicles that are constituted by an aqueous core surrounded by a single bi-layer of surfactant, and the terms "paucilamellar" or "plurilamellar" to designate vesicles having a plurality of bi-layers. Multilamellar vesicles include those referred to below as "conventional type multilamellar vesicles" (MLV), and vesicles of very particular structure referred to below as "onion-structure" multilamellar vesicles. These two types of multilamellar vesicles are distinguished by three fundamental differences.

1. <u>The method of obtaining them</u>

Conventional MLVs are generally obtained by using a preliminary mixture in an organic solvent medium of lipids and other components constituting the envelopes of said MLVs, and then evaporating off the solvent so as to obtain a dry film. The vesicles are then obtained by rehydrating the lipid film using an aqueous solution containing the active agent to be encapsulated.

Onion-structure multilamellar vesicles are obtained by applying shear to a liquid crystal lamellar phase including the active agent to be encapsulated.

2. <u>Their structure</u>

Because of the way they are prepared, conventional MLVs are aggregates of lipid type multilamellar lamellae gathered together within an approximately spherical lipid membrane. This structure can be seen very clearly in the photograph in US patent 4 975 282.

Liposomes are usually obtained form the above-described conventional MLVs after applying a very high degree of shearing (by ultrasound or a French press), transforming them into unilamellar or plurilamellar vesicles characterized by the presence of an aqueous core.

Onion-structure multilamellar vesicles are in the form of a regular stack of concentric bi-layers going from the very core of the vesicle to the periphery.

3. Their thermodynamic nature

Inside conventional multilamellar vesicles, the number of multilamellar lamellae, the numbers of layers they have, the numbers of folds, and the way in which they are arranged are characteristics which depend on the method of preparation and in particular on kinetic phenomena that occurred during rehydration of the lipid film. The internal structure of the vesicles is not uniform within a vesicle (zones with little curvature, zones of great curvature, multilamellar zones, continuous zones). Such a structure is thus not in thermodynamic equilibrium. In addition, each vesicle is of a different constitution, so the structure thereof it not uniform across an entire sample.

Liposomes of conventional type having a few layers are described in particular in application WO 96/31194, which liposomes are constituted by a few layers surrounding an aqueous core, within which there are to be found in association a molecule and its stabilizer.

Compositions in which an active agent is encapsulated within multilamellar vesicles of onion-structure and constituted from the center all the way to the periphery by a succession of lamellar layers separated by a liquid medium are already described in the various patents referred to below. These vesicles can be obtained by a method including preparing a liquid crystal lamellar phase and transforming it by applying shear.

Such a method is described in particular in patent WO 93/19735 derived from French patent FR 2 689 418, or WO 95/18601 which are included herein by reference.

According to French patent FR 2 689 418, this transformation can be performed during a step of applying uniform shear to the liquid crystal paste, which leads to vesicles of controlled size, also known as "microcapsules". Nevertheless, by varying the formulation of the liquid crystal lamellar phase, in particular the nature of the surfactants used in making it up, this liquid crystal phase can be transformed into vesicles merely by applying mechanical force thereto, in particular while mixing together its components.

Various applications for vesicles of that type have been described by the Applicant. Particular mention can be made of international application WO 95/19707 which describes odorous compositions in which an odorous substance is incorporated within such multilamellar vesicles, with the effect of such encapsulation being to increase the staying power of the odor, by slowing down evaporation. Mention is also made of French application FR 2 735 658 which describes compositions for food use in which a substance or an additive for food use is included within such multilamellar vesicles, with the essential effect of obtaining highly advantageous controlled release of the encapsulated product, the presence of the multilamellar vesicle also making it possible to protect often-fragile molecules that are incorporated therein prior to their inclusion in the compositions.

Nevertheless, even though such a composition does under some circumstances produce an already-perceptible effect of stabilizing fragile molecules, it can turn out to be insufficient for the highly sensitive molecules for which particularly strong stabilization is desired against the effects of an environment that is unfavorable, a priori.

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Thus, the inventors of the present invention have now discovered that the already-observed action of protecting fragile molecules, in particular certain substances in the food industry, can be considerably increased by incorporating an agent for stabilizing such fragile molecules within onion-structure multilamellar vesicles. Presenting a pair of agents comprising an active agent and a stabilizing agent in this manner makes it possible to obtain a higher degree of stabilization, and thus to obtain a longer lifetime for products incorporating the active agent, and to do so with the stabilizing agent at concentrations that are clearly lower, thereby constituting a considerable advantage of the present invention.

This invention is particularly advantageous in any field where it is desired to protect an active agent from a degrading agent. It applies to all substances known for their fragility, and most particularly to vitamins and to enzymes.

With enzymes, the invention provides a particularly effective means for ensuring that the enzyme is immobilized and simultaneously protected from the surrounding medium in order to improve its stability.

Thus, the present invention provides a particularly economic and effective solution to the problems associated with stabilizing fragile active materials, together with all of the advantages associated with the encapsulation technique used:

- great flexibility in formulation;
- a wide variety of suitable surfactants;
- the ability to implement a plurality of active agents simultaneously;
- the possibility of using a plurality of agents for the purpose of further improving stabilization; and/or
- greater stability in an aqueous medium, whether on its own or in association with some other medium (gel, detergent, emulsion).

Thus, according to one of its essential characteristics, the invention provides compositions containing an active agent encapsulated within multilamellar vesicles presenting an onion-structure and constituted, from their peripheries to their centers, by concentric membranes in the form of bi-layers comprising at least one surfactant, said membranes being separated by an interstitial liquid, said vesicles containing at least one agent intended to avoid the degradation of said active agent.

As explained above, the term "onion-structure" is used to mean a multilamellar structure in which vesicles of substantially spherical shape are constituted by a succession of concentric bi-layers, with this extending from the center of a vesicle to its periphery, whence the term "onion-structure" which is used by analogy to describe such structures.

These structures can be observed by examining the compositions under the microscope. Observation is performed using an optical microscope with polarized light, such that birefringence is observed in a lamellar phase. This manisfests itself by a characteristic texture associated with the presence of defects (grain boundaries) between domains having phases that are oriented differently. With the vesicles in a concentrated phase, the texture is characterized by its fine and uniform nature, associated with the size of the vesicles. With the vesicles in a dispersed phase, they are visible as slightly birefringent points that are more or less well resolved (depending on size). Birefringence can be observed only when the dispersion is not too dilute. Thus, if the dispersion is quite dilute, it is appropriate to proceed with a prior operation of concentrating the solution to show up clearly the birefringence that is characteristic of the presence of onion-structure vesicles.

The principle of the invention consists in using vesicles as microreceptacles containing the molecule to be protected, and preventing the degradation reaction from taking place. For this purpose, the vesicle performs two roles: firstly, it isolates the active molecule from its environment, and secondly it provides additives necessary for stabilizing it, which turns out to be particularly advantageous with sensitive molecules. One of the major advantages of the vesicle is to confine the fragile molecule and to protect it in a volume that is small, much smaller than the total volume of the preparation, thereby avoiding the effect of dilution, which makes it possible as a result to use only a small quantity of the protecting molecule.

In an advantageous variant, the interstitial liquid is water and the active agent is included in the membranes of said vesicles when it is hydrophobic or in the interstitial liquid when it is hydrophilic.

In another advantageous variant of the invention, the vesicles are of dimensions lying in the range 0.1 µm to 50 µm, and preferably in the range 0.2 µm to 10 µm.

Such structures are advantageously obtained by incorporating the active agent and the agent for stabilizing it in a liquid crystal lamellar phase having at least one surfactant and then in transforming said lamellar liquid crystal phase into a dense phase of multilamellar vesicles of small size by applying shear thereto.

The shear may be uniform shear, which presents the advantage of leading to vesicles that are entirely uniform in size. Nevertheless, mere mechanical stirring can be sufficient for causing multilamellar vesicles of the invention to be formed.

According to French patent FR-2 689 418, this transformation can take place during a step of applying uniform shear to the liquid crystal phase, thereby leading to vesicles or microcapsules of controlled size.

Nevertheless, by varying the formulation of the liquid crystal lamellar phase, in particular by varying the kinds of surfactants used in making up its composition, it can be possible to transform the liquid crystal phase into vesicles merely by applying mechanical force, in particular while mixing its components.

As can be seen in particular from the examples illustrating the invention, the choice of surfactants suitable for use for forming the multilamellar vesicles of the invention is very wide. Nevertheless, the surfactants should be selected as a function of the intended field in which the composition is to be used. In numerous cases, the intended application has constraints which put a limit on the surfactants that can be selected. These are often legal constraints or constraints associated with standards. Thus, in the field of cosmetics, the international nomenclature of cosmetic ingredients (INCI) catalog provides the list of substances that are authorized, and in the food industry, reference should be made to the positive list of authorized additives, and in the field of pharmacy, reference should be made to the pharmacopeia.

The formulation advantageously makes use of a mixture of surfactant molecules. In general, at least two different surfactants are used having different hydrophilic-lipophilic balances, thereby making it possible to adjust continuously the properties of the bi-layer and thus control the appearance of instability which governs the formation of multilamellar vesicles.

In a particularly advantageous variant of the invention, a mixture of two surfactants is used, referred to respectively as a lipophilic surfactant having a hydrophilic-lipophilic balance (HLB) lying in the range 3 to 7, and as a hydrophilic surfactant having an HLB lying in the range 8 to 15.

In another advantageous variant of the invention, the membranes of the vesicles contain at least one

polymer surfactant or a surfactant comprising a polymer having amphiphilic properties.

This applies in particular to poloxamers and other copolymer derivatives of ethylene oxide and propylene oxide, optionally modified by adding hydrophobic chains.

By way of non-exhaustive examples, mention can be made of the Pluronic® and Lutrol® (BASF) families, and of polyethylene hydroxystearates (Soluton® from BASF or MYRJ® from ICI).

The invention applies in particularly advantageous manner to any type of active ingredient known for its fragility. This applies in particular to agents that are sensitive to oxidation or to reduction, to hydrolysis or to more specific reactions such as autoproteolysis in the case of proteases.

Particular fragile active agents to which the present invention applies more specifically include reducing and oxidizing molecules that are sensitive to hydrolysis, in particular vitamins, enzymes, proteins, and biological molecules in general.

A field in which the invention has particularly advantageous applications is that of insecticides. There are several large families of insecticides including organophosphates, organochlorides, and pyrethroids. The present trend is to use molecules that are sufficiently biodegradable to enable them to be destroyed quickly in the environment. For example, organochlorides are nearly all banned because they persist for too long in the environment. This gives rise to the molecules that are used having a problem of the stability over time. This problem is raised by certain organophosphates which are hydrolyzed quite quickly, or by pyrethroids (synthetic derivatives of pyrethrin, a chrysanthemum extract) which are unstable. The method of the invention is particularly advantageous for stabilizing all such particularly unstable pyrethroids.

Another field in which the invention has particularly advantageous applications is that of cosmetics and dermatology where numerous active agents are known for being fragile. This applies in particular to vitamins A, E, and C, for example, and also to molecules such as DHA (dihydroxyacetone, a self-tanning agent), to procyanidolic oligomers, and to enzymes.

Another field in which the invention is particularly applicable is that of medicaments in which the concepts of molecule fragility, and of limits on authorized additives are even more pertinent than in cosmetics.

Another example where the invention has particularly advantageous applications is that of stabilizing hydroquinone and its derivatives which are substances in widespread use in the field of photography as a developer, and also in cosmetics where they suffer most particularly from sensitivity to oxidation because of their reducing properties.

The agent for stabilizing the active agent should be selected as a function of the nature of the active agent and as a function of the type of degradation that is to be avoided, with this also taking account of the type of application intended.

Thus, the additive(s) necessary for stabilization purposes can be one or more compounds specially designed to provide protection to the active agent. This applies when the active agent is a substance that is sensitive to oxidation and the agent for avoiding degradation thereof is an agent known as an anti-oxidizing agent.

This also applies when the active substance has its stability modified by a variation in pH which can give rise, for example, to a hydrolysis reaction or to a modification in the redox potential of the active substance. Under such circumstances, it is appropriate to encapsulate a stabilizing agent that makes it possible to modify pH locally, so as to increase the stability of the active substance.

When it is desired to improve the stability of an active substance that is sensitive to oxidation, the vesicle advantageously contains a substance known for its anti-oxidant properties because of its reducing properties or because of its action in decreasing the risk of oxidation by a trapping effect, e.g. by an effect whereby it traps traces of oxidation-catalyzing metal ions contained in the medium, or by its action on the pH of the medium when the redox potential depends on pH.

Thus, and in non-exhaustive manner, the following can be mentioned as anti-oxidizing agents:

- ascorbic acid and derivatives thereof;
- citric acid and derivatives thereof (also used as a trapping agent);
- glutamic acid, glutamates, and derivatives thereof;
- ethylene diamine tetraacetic acid (EDTA) (trapping agent);
- lactic acid and derivatives thereof (also used for adjusting pH);
- tartaric acid and derivatives thereof (also used for adjusting pH and as a trapping agent);
- benzophenone;
- bioflavonoides;
- butylhydroxy hydroxyanisol;
- butylhydroxy hydroxytoluene;
- carotene and derivatives thereof;
- chlorobutanol;
- propyl, octyl, or dodecyl gallates;
- sodium or potassium sulfite, and related compounds such as bisulfites or pyrosulfites;
- (alpha, delta, or gamma) tocopherols and derivatives thereof; and
- in general terms all food additives in classes E3xx and E22x in the European classification of food additives.

By way of example, vitamin C and derivatives thereof are well known for their sensitivity to oxidation. It has been shown clearly that the stability thereof can be improved by co-encapsulating them with an anti-oxidizing agent.

As the anti-oxidant, it is possible to use sodium bisulfite and/or a trapping agent for eliminating traces of oxidizing metals, for example ethylene diamine tetraacetic acid (EDTA).

An example of an agent suitable for avoiding hydrolysis by the effect of locally modifying pH, mention can be made of sodium tartarate which can be co-encapsulated with magnesium ascorbylphosphate (APG) in order to increase its stability by locally modifying pH.

The agent for stabilizing the active substance can also form a part of the membrane of the vesicle if it has amphiphilic properties. This applies to vitamin E or derivatives thereof which can be co-encapsulated with an active agent that is sensitive to oxidation, so as to play an anti-oxidizing role and thus a protective role. However vitamin E (tocopherol acetate) is an amphiphilic substance given the nature of its molecule. Advantage can therefore be taken of this property to adapt the formulation of the liquid crystal phase. Thus, vitamin E is incorporated in the surfactant membrane and plays an active role in forming said membrane, enabling suitable characteristics to be imparted to the liquid crystal phase for enabling vesicles to be obtained by shear.

Other cases also exist in which the stabilizing agent likewise plays the role of a surfactant contributing to forming vesicle membranes. Such examples are given below with reference to stabilizing enzymes.

In another variant of the invention, it is possible for the agent for stabilizing said active agent to be selected as an agent which, itself, constitutes a second active agent of the composition. This applies for example to vitamin E which, because of its anti-radical

properties, constitutes, in a cosmetic composition where it is associated with vitamin A or with vitamin C, not only an agent for stabilizing the vitamin A as described above, but also a second active agent of said composition.

In the particular case where the molecule to be stabilized is an enzyme, the stabilizing agent will, in general terms, either be an additive known for stabilizing or protecting proteins, referred to below as a non-specific enzyme-protecting additive, or else a specific agent, specifically for stabilizing one enzyme in particular.

Thus, with proteases, a protease inhibitor can avoid autoproteolysis.

In a first variant where a non-specific protective additive is used, the additive is advantageously selected from substances which are known for acting on the conformation of the enzyme. As an example of such substances, particular mention can be made of ions having the effect of increasing ionic force and of fixing themselves on certain charged sites of the enzyme, or of molecules suitable for engaging weak bonds with the protein.

The person skilled in the art will readily understand that the most effective ions are relatively large ions, e.g. ammonium ions and quaternary ammonium ions for cations, and sulfates, phosphates, carboxylates, and carboxylic polyacids for anions. The person skilled in the art will easily understand that best effectiveness is obtained by selecting ions that are large enough or that are attached to a molecule that is itself large enough.

Ions that are particularly advantageous for stabilizing enzymes include the calcium ion which is well known to intervene specifically in the reactivity of many enzymes, in particular of proteases.

In the variant in which specific stabilizing molecules are used, it is advantageous to select molecules that carry functions suitable for bonding with the enzyme, e.g. molecules that are capable of forming hydrogen bonds with the enzyme. Such molecules include in particular alcohols and polyols, advantageously polyols associated with a derivative of boron, in particular a borate ion, ethoxylated amines, and amine oxides. It is also possible to make use of surfactants that include a plurality of ethylene oxides. Such surfactants can be used in formulating the membranes of vesicles of the invention and participate in stabilizing enzymes.

Thus, as agents suitable for stabilizing enzymes and incorporated within such vesicles, mention can be made of surfactants and amphiphilic molecules that contain the following functions or substituted by the following groups:

- quaternary ammoniums;
- amines and ethanolamine;
- molecules carrying a phosphate function, in particular phospholipids;
- salts and esters of fatty acids;
- salts of polyacids;
- alcohols;
- glycerol and esters thereof (glycerides);
- polyols (polyglycerides, polyethyleneglycol, polypropyleneglycol); and
- sugars (sorbitol, glucose, lactose, saccharose...).

The enzyme-stabilizing agents are advantageously selected from polymer derivatives containing functions of one of the above types, and in particular:

- optionally modified polysaccharides such as:
  - agarose,
  - guar gums,
  - carrageenans,
  - alginic acid and alginate,

- pectin, and
- chitosan;
- optionally substituted polyvinylpyrrolidones;
- cellulose and (alkylated or functionalized) cellulose derivatives;
- polyacrylates;
- polyvinylalcohols (PVA) and partially hydrolyzed derivatives of polyvinylacetates;
- polyacrylamides; and
- polyamides.

Tests performed by the inventors of the present invention have shown that the presence of either a surfactant or an amphiphilic molecule having at least one nitrogen-containing function, or else of a polymer that likewise presents a nitrogen-containing function as an agent for avoiding degradation of an enzyme in multilamellar vesicles that incorporate said enzyme, make it possible to significantly improve the stabilization of the enzyme.

When a surfactant having at least one nitrogen-containing function is used as the agent for stabilizing an enzyme, it is preferable to select a surfactant in which the nitrogen-containing function forms a portion of the polar head of said surfactant.

In this variant where the nitrogen-containing functions form a portion of the polar head of the surfactant, the hydrophobic tail is advantageously constituted by one or more carbon-containing chains. It is thus possible to define independently the nature of the hydrophobic portion which does not vary much from one surfactant to another: at least one (in general two) alkyl chains having 1 to 22 carbon atoms, which may be linear or branching, simple or substituted, optionally carrying a cyclic or an aromatic residue, saturated or carrying one or more points of non-saturation, possibly substituted by other functions. The chain(s) forming the hydrophobic portion of the surfactant can either be

directly bonded to the nitrogen atom of the nitrogen-containing function, or else, more preferably, can be bonded to one of the nitrogen substituents.

When a polymer carrying at least one nitrogen-containing function is used to stabilize an enzyme, it is advantageous to select from the following list:

· polyacrylamides and products of polymerizing or copolymerizing derivatives of acrylamide;

· amide-containing derivatives of polysaccharides, in particular quaternized guar gums such as hydroxypropyltrimonium guar chloride;

· derivatives of chitin such as chitosan, salts of poly D-glucosamine, counter-ions of such polymers possibly containing an amine function or a derivative thereof (e.g. glutamate); and

· polyamides.

In the specific case in which it is desired to stabilize an enzyme to avoid autoproteolysis thereof, it is advantageous to co-encapsulate a protease inhibitor, e.g. EDTA, phenylmethanesulfonylfluoride, 3,4-dichloroisocoumarin, or chymostatin.

In order to reinforce the confinement role played by the vesicle, it can be advantageous for its formulation to include one or more polymers or molecules having a high melting point, thereby reinforcing the leakproofing of the vesicle. This improved leakproofing can also be obtained by any means enabling interchanges with the final dispersion medium to be decreased, in particular by coating the vesicle with a polymer or a wax, possibly an auto-emulsifier wax. Thus, in an advantageous variant, the invention also covers compositions in which the vesicles further comprise at least one agent for reinforcing their leakproofing, said agent being encapsulated within the vesicles or constituting an external coating of said vesicles.

In a particularly advantageous variant of the invention, it is possible to select as the agent for

stabilizing the active substance, an agent which simultaneously improves the leakproofing of the vesicle. Thus, to stabilize vitamin C, it is co-encapsulated with pectin which, by binding to vitamin C, stabilizes it and simultaneously improves the leakproofing of the vesicle.

To sum up, the invention consists in using a multilamellar microvesicle as a confinement medium for a fragile molecule together with additives for stabilizing the fragile molecule, while possibly also reinforcing the leakproofing of the microvesicle.

According to another aspect, the invention also provides a method of preparing a composition as defined above, the method comprising the steps of:

· preparing a liquid crystal lamellar phase comprising at least one surfactant and incorporating at least one active agent and an agent for avoiding degradation of said active agent; and

· transforming said liquid crystal phase into multilamellar vesicles of onion-structure by shear.

This shear is advantageously uniform shear as taught in patent FR 2 689 418.

Transforming the liquid crystal lamellar phase into onion-structure multilamellar vesicles can be performed by selecting the surfactants. In particular, as mentioned above, it is possible to select one surfactant having high HLB and another surfactant having low HLB.

Furthermore, certain surfactants are particularly suited for favoring such transformation. This applies for example to poloxamers and other copolymer derivatives of ethylene oxide and propylene oxide possibly modified by adding hydrophobic chains. These compounds are particularly advantageous since they serve both to match the elastic properties of the surfactant membrane and to stabilize it by the steric hindrance effect. Mention can be made by way of non-exhaustive examples of the Pluronic® and Lutrol® (BASF) family, and of polyethylene hydroxystearates (Solutol® of BASF or MYRJ® of ICI).

Finally, in a last aspect, the invention also provides a method of improving the stability of an active substance and of avoiding degradation thereof, the method being characterized in that it consists in encapsulating said active substance within multilamellar vesicles as defined above, having an onion-structure and constituted, from the periphery to the center, by membranes in the form of concentric bi-layers comprising at least one surfactant, said membranes being separated by an interstitial liquid, said vesicles incorporating within them at least one agent for avoiding degradation of said active agent.

For the reasons mentioned above, the invention has a particularly advantageous application in stabilizing and/or immobilizing enzymes.

Thus, the invention relates most particularly to a method of protecting and/or immobilizing an enzyme whereby said enzyme is encapsulated within multilamellar vesicles of onion-structure as defined above, said vesicles containing within them at least one agent as defined above for the purpose of avoiding degradation of said enzyme.

The excellent results have been obtained concerning enzyme stabilization when the enzyme is incorporated within a multilamellar vesicle having at least one stabilizing agent selected from surfactants, amphiphilic molecules, and polymers, carrying at least one function capable of bonding to said enzyme, appear clearly to be associated with the existence of a bond whereby the enzyme is, so to speak, complexed with one of the components of the vesicle membrane. These results suggest an interaction of the same type could occur between the surface of a multilamellar vesicle incorporating such a stabilizing agent in its membranes. This hypothesis has been confirmed by the fact that it has also been possible to observe a clear stabilizing effect on an enzyme when it is put into contact within a

composition with multilamellar vesicles of onion-structure of a formulation that is such that the membranes comprise at least one stabilizing substance selected from amphiphilic molecules, surfactants, and polymers, carrying at least one function suitable for bonding to said enzyme.

Thus, in another aspect of the present invention, it also provides a method of protecting and/or immobilizing an enzyme whereby said enzyme is put into the presence of onion-structure multilamellar vesicles, i.e. vesicles that are constituted from the periphery to the center by membranes in the form of concentric bi-layers having at least one surfactant, said membranes being separated by an interstitial liquid, said vesicles incorporating within them at least one stabilizing agent compound carrying at least one function suitable for bonding with said enzyme. The stabilizing agent is selected from the above-mentioned amphiphilic, surfactant and polymer molecules that have a stabilizing effect on an encapsulated enzyme.

The following examples are given purely to illustrate the invention and they show how it is possible with the invention to improve the stability of various active agents that have the reputation of being fragile.

These examples also make reference to Figures 1 to 4:

· Figure 1, given with reference to Example 1, gives variation in the concentration of non-degraded vitamin C as a function of time for a composition in which the vitamin C is encapsulated, compared with a composition in which it is free;

· Figure 2, given with reference to Example 3 shows how magnesium ascorbylphosphate (APG) is stabilized by co-encapsulation with an agent that modifies its pH;

· Figure 3, given with reference to Example 5, shows how trypsin is stabilized by being encapsulated in onion-structure multilamellar vesicles containing, as a

stabilizing agent, a surfactant carrying an ammonium function; and

Figure 4, given with reference to Example 6, shows how trypsin is stabilized by being encapsulated in an onion-structure multilamellar vesicle incorporating a surfactant of the esterquat family.

EXAMPLES

In the examples below, unless stated otherwise, proportions are given as percentages by weight.

Example 1

Vitamin C

Vitamin C is very sensitive to oxidation. It has been stabilized by being encapsulated in multilamellar microvesicles reinforced by a natural cross-linked polymer (pectin) and by co-encapsulating additives that enable stabilization: a trapping agent that removes traces of metal ions that catalyze oxidation (EDTA); and an anti-oxidant (sodium bisulfite) by proceeding as follows:

a) Formulation

Components A	%
Polysorbate 60	24
Sorbitan oleate	20
Acetate of vitamin E	5
Preservative	1
Components B	%
Water	37.5
Ethylenediaminetetra acetic acid	0.08
Sodium bisulfite	0.82
Pectin	1.6
Ascorbic acid	10

b) Procedure

Ingredients B were mixed for 30 minutes (min) at ambient temperature under stirring and in the same order as in the list. A translucent gel was obtained.

Ingredients A were mixed at ambient temperature under stirring, and then mixture B was added while maintaining stirring at ambient temperature. Stirring was maintained for 2 hours (h).

A thick paste was obtained that needed to be dispersed to 50% in a solution of calcium chloride to cross-link the pectin. For that purpose, an aqueous solution containing 20 grams per liter (g/l) of $CaCl_2$ was added slowly to the paste maintained under stirring. A milky fluid dispersion was obtained and under the microscope it was observed to show the presence of birefringent vesicles.

c) Determination

The dispersion of vesicles was rediluted in water to achieve a vitamin C concentration of 5%. The dispersion was placed in an oven at 45°C to monitor its stability. The vitamin C content in the sample was measured by iodine assay.

The result of the stabilization is shown by the curve in Figure 1 giving vitamin C content as a function of time at 45°C for the vesicle dispersion (marked "encapsulated" in Figure 1) prepared as described above, and in comparison with the results obtained for a simple dispersion of vitamin C having the same initial concentration in water (marked "free" in the figure).

It will be observed that whereas the vitamin C solution degraded continuously, the encapsulated vitamin C remained at 80% of its initial quantity for more than 45 days at 45°C.

Example 2

Vitamin A and vitamin E

a) Formulation

	Component	%
A	Sucrose palmitate	40
B	Glycerol linoleate	9
C	Vitamin A palmitate	15
D	Vitamin E acetate	1
E	Water	34
F	Preservative	1

b) Procedure

B, C, D, and F were mixed at ambient temperature for 10 min. Thereafter A was added carefully while maintaining stirring. Once A had been fully incorporated, E was added and then the temperature was raised to 65°C and maintained for 1 h. The temperature was then lowered to 40°C while maintaining stirring over a time interval of 2 h.

A uniform birefringent paste was obtained which under the microscope displayed characteristic fine and regular texture.

c) Results

HPLC monitoring showed clearly that the stability of vitamin A had been improved because of its encapsulation in vesicles also containing vitamin E.

Example 3

Magnesium ascorbyl phosphate (APG)

This compound is a derivative vitamin C that is more stable than ascorbic acid, but that is nevertheless not very stable at a pH of less than 7.5. This gives rise to problems in cosmetics since the pH of the skin is 5.5,

and it is preferable for the pH of a cosmetic to be close to that of the skin.

Under such circumstances, encapsulation was performed in microvesicles of non-ionic surfactants. APG was initially dissolved in a sodium tartrate medium, co-encapsulated in the vesicles and providing stabilization, by proceeding as described below. The curves showing degradation of free and encapsulated APG at 45°C and at pH = 5.5 are given in Figure 2 which shows the percentage of non-degraded APG as a function of time.

a) Formulation

Component	%
Polysorbate 60	12
Sorbitan stearate	34
Tocopherol acetate	4
Solution A	49
Preservatives	1

Solution A:

Tartaric acid:	2.88%
APG:	28%
Water:	69.12%

b) Procedure

Solution A was prepared by previously dissolving tartaric acid in water, adjusting the pH to 7.3-7.5 by adding 6N caustic soda, and slowly adding the APG in powder form under stirring at ambient temperature.

The surfactants, the vitamin E, and the preservative were mixed under stirring at 70°C. Once the mixture had dissolved and was uniform, solution A was added slowly under stirring at said temperature. Once incorporation was complete, heating was stopped, and the temperature allowed to fall to ambient, under stirring, over 1 h.

c) Stability

The stability of the product was monitored by iodine assay of the vitamin C in a 3% aqueous dispersion of APG, either free or encapsulated in multilamellar vesicles. The results are given in Figure 2.

It can be seen that encapsulated APG degrades at one-tenth the rate of free APG at this pH (by measuring degradation half-life).

Example 4

Hydroquinone and derivatives thereof

Hydroquinone and its derivatives are commonly used in photography as a developer, and also in cosmetics as an agent for lightening the skin. These molecules are highly reducing, and therefore oxidized easily in air or in an aqueous medium.

Methylhydroquinone was encapsulated in vesicles of non-ionic surfactants encapsulating sodium bisulfite as an anti-oxidant.

a) Formulation

Vesicles

Component	%
Polysorbate 60	10
Sorbitan stearate	40
Glycerol	34
Methylhydroquinone	16

Dispersion

Component	%
Vesicle paste	50.0
Sodium bisulfite	0.05
Preservative	0.8
Softened water	49.15

b) Procedure

All operations (preparation, transfer, and dispersion) were performed under an argon atmosphere.

The methylhydroquinone was initially dissolved in glycerol to a concentration of 32% methylhydroquinone for 68% glycerol by mixing at 90°C for 30 min.

The two surfactants were mixed together and heated to 70°C in an emulsifier provided with mechanical stirring using a scraper blade. Once the surfactants had melted, the solution of methylhydroquinolone in glycerol was added and the mixture was maintained at said temperature for 30 min. Heating was then stopped to allow the temperature to return to ambient under stirring. A uniform white paste was obtained and under the optical microscope in polarized light it could be seen to have the birefringent texture characteristic of the presence of microvesicles.

Dispersion was performed by slowly adding the dispersion solution (degassed water and sodium bisulfite) to the above paste in an Erlenmeyer flask fitted with a mechanical stirring blade, at ambient temperature and under continuous stirring. The preservative was added on a single occasion at the end of adding the aqueous solution. Stirring was maintained for about 2 h to obtain complete dispersion. Observation under an optical microscope showed a dispersion of slightly birefringent vesicles.

c) Results

The encapsulated product showed only slight pink coloring while a solution of methylhydroquinone became dark quickly.

Example 5

Stabilizing an enzyme by encapsulation in a vesicle containing a surfactant carrying an ammonium function

1) The principle of the test for showing enzyme stabilization

To demonstrate the stabilizing effect on enzymes, the activity of an encapsulated enzyme was measured as a function of aging time by comparison with an enzyme merely put into solution and under the same conditions of aging. Since the encapsulated enzyme is not accessible, it was necessary to begin by releasing it in order to be able to measure its activity. The experiment thus had four stages:

* preparing samples
* encapsulating the enzyme and dispersing the vesicles containing the enzymes;
* putting the same concentration of a control enzyme into solution
* aging the dispersion of vesicles and the control solution;
* releasing the enzyme from the vesicles; and
* measuring the enzymatic activity of the sample under test and of the control sample.

Enzymatic activity was evaluated by a conventional method of tracking the degradation reaction of a substrate characteristic of the enzyme.

2) Test conditions

a) Enzyme

The enzyme used was trypsin, i.e. a serine protease (like the proteases used in detergents). Its mass was 23,000 grams per mol (g/mol) and its size lay in the range 20 Å to 40 Å.

Packaged in freeze-dried form, it was dissolved in a buffer in the form of a 4% by weight solution. This is the 4% solution that was used in the preparations.

For measurement purposes, the control samples were prepared using 50 microliters (µl) (50 mg) of the solution per 5 g of final solution, giving an enzyme content in the final solution of 0.04% by weight.

b) Vesicles

The enzyme content of the vesicles was 0.15% by weight. Unless stated otherwise, the vesicles were prepared by mixing the surfactants at ambient temperature and then incorporating the aqueous solution of the enzyme. A viscous paste was thus obtained, of characteristically birefringent texture (observed under an optical microscope in polarized light). This paste was dispersed by slowly adding water while stirring at ambient temperature.

To measure activity, all of the vesicle dispersions had a 2% vesicle content. The enzyme content of the vesicle dispersion was therefore 0.04%.

c) Buffer

This was a phosphate buffer having the following composition:

NaCl:	8 g/l
KCl:	0.2 g/l
Na_2HPO_4:	1.15 g/l
KH_2PO_4:	0.2 g/l

Its pH lay in the range 7.5 to 8.

d) Substrate

N-benzoyl L arginyl ethyl ester (BAEE) was used as the substrate, i.e. a substrate that is conventionally used for studying the activity of trypsin insofar as decomposition thereof is catalyzed by trypsin. The reaction product absorbs in the ultraviolet (UV).

e) Determining activity

This was performed as follows:

e1) Destroying the vesicles

25 µl of sample were taken and 20 µl of X100 triton solution at 10% (from Sigma) were added. It was allowed to act for 30 seconds.

e2) Measuring enzyme activity

37 µl of substrate solution at 0.025 mol/l were taken, and 950 µl of buffer and 22.5 µl of sample were added thereto. Reaction kinetics were monitored for 10 minutes at 20°C using a wavelength of 253 nm.

The samples were aged in an oven at a temperature of 37°C.

3) Tested formulation

Surfactants used:

Polysorbate 60: polyoxyethylene (20) sorbitan monosterate

DODMAB: dioctadecyl dimethyl ammonium bromide

Formulation:

- 15% aqueous solution of enzyme
- 35% water
- 25% DODMAB
- 25% polysorbate 60

Preparation:

The surfactants and the enzyme solution were mixed coarsely at ambient temperature and then the mixture was put into a Couette type cell using the procedure disclosed in patent WO 93/19735.

Shearing took place at 25°C for 20 minutes, at a rate lying in the range 50 s^{-1} to 100 s^{-1}.

4) Results

Enzyme degradation was followed by UV spectroscopy.

Figure 3 shows the variations in enzyme activity over time for the encapsulated enzyme in comparison with the variation observed for the free enzyme. Initial activity was arbitrarily fixed at 100 for plotting the curve.

Figure 3 shows clearly that the enzyme was stabilized by being encapsulated.

Example 6

Stabilizing an enzyme by encapsulating it in a vesicle containing an esterquat

The procedure was the same as in Example 5. The surfactant used in this example was taken from the family of esterquats. It was: N, N di ("acyl" oxy-2-ethyl), N-hydroxy-2-ethyl, N-methyl ammonium methosulfate in solution in isopropanol as sold by CECA under the trademark Noxamium 920.

Formulation (percentages by weight):

aqueous solution of enzyme	15%
water	35%
Noxamium 920	50%

Preparation

The vesicles were prepared in a manner analogous to that of Example 5.

Figure 4 shows variations in enzyme activity over time for the encapsulated enzyme compared with the variations observed for the free enzyme. Initial activity was arbitrarily set at 100.

This figure shows clearly that the enzyme was stabilized by being encapsulated.